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1 October 2004

FAX Nº: 00 49 89 2399 4465

Reply to the written opinión pursuant Rule 66 PCT

Re: INTERNATIONAL PATENT APPLICATION NO. PCT/ES03/00363

Dear Sirs,

We respond in due time, after the extension term kindly granted by that office to reply, by enclosing hereto a new set of amended claims that, in our view, overcomes the general objection raised by the examiner in charge of present International Application (IA).

1.- Support for the amendments made

In our view, no fresh technical matter has been introduced with the amendments made into the claims, with regard to the IA as formerly filed. The amendments made are more related to a reorganization of the different claim categories (products, processes and uses) with some minor re-drafting work than to a substantial scope change.

Amended claim 1: It is the result of combining former claim 12 with the preamble and the essential feature (phosphodiesterase activity) of former claim 3 from which formerly depended on .

Amended claim 2: It is the result of redrafting former claim 13, limited according to the cDNAs of former claims 16 and 18.

Amended claim 3: It is the result of redrafting former claim 19

Amended claim 4: It is the result of redrafting former claim 20

Amended claims 5-13: They are the result of redrafting former claims 3-11

Amended claim 14: It is the result of redrafting former claim 1, limited to the tryptic amino acid sequences identifying the enzyme as formerly present in former claim 12.

Amended claim 15: It is the result of redrafting former claim 2

Amended claims 16-20: They are the result of redrafting former claims 14-18

Amended claims 21-33: They are the result of redrafting former claims 21-33 according to the new dependency scheme.

As you may notice the only purpose of the minor changes introduced it has been to differentiate the NPPase of present IA from the AGPPase previously disclosed in the prior art. We agree with the examiner that, by using only essential features defining the catalytic activity of both enzymes, they seem to be the same. However the inventors have discovered that the AGPPase previously protected by WO0157196 (also in the name of the applicant) and further disclosed in the PNAS scientific article (whose authors are also the inventors) was really a regulatory subunit of an enzymatic complex wherein the NPPase, the enzyme now claimed in the present invention, shows the catalytic activity.

2.- Unity of the invention

The amended set of claims, in our view, has a single inventive concept which would comprise different categories of claims. The single inventive concept it is the characterization of a new enzyme by means of either functional catalytic or structural (Amino acid sequence, cDNA, tryptic fragments,...) features. Accordingly, the following claim categories integrate the single inventive concept:

- Product claims covering the new enzyme (1-13)
- Process for isolation the new enzyme from plant sources (14-15)
- Uses of the new enzyme (21, 27 and 28) and kits where the enzymes are used thereof (22-26; 33)
- Process for production transgenic plants which over-expresses the enzyme (29-31), transgenic plants produced thereof (32) and primers and cDNAs used in said process (16-20)

3.- Novelty

The NPPase enzyme as now claimed is clearly not he same than the AGPPase previously disclosed in the prior art cited in the WO. In spite of the above mentioned fact that both enzymes AGPPase and NPPase form part of the same enzymatic complex. This is the reason why, when isolated from plant sources, the catalytic measurements, as far as substrates to be hydrolysed or ligand requirements are the same. The isolation always comprises a percentage of each subunit, either the regulatory (AGPPase) or the catalytic (NPPase). However, present set of amended claims now cover only NPPase as a different enzyme from the previously disclosed AGPPase disclosed in the prior art as proves their different tryptic sequences and MWs (70 and 270 kDa for NPPase monomeric and homopolymeric isoforms, see amended claim 12 vs. 35-55 kDa for AGPPase, see claim 10 of WO0157196) . Moreover, NPPase is a glycoprotein (see pg. 18 line 12) and AGGpase was not.

4.- Inventive step

The objective problem solved in present set of amended claims is to find an alternative enzyme showing catalytic functional activities to the AGPPase disclosed in the prior art. When we expressed in transgenic plants the DNA construction encoding for NPPase (catalytic activity) we have observed enzyme activities of 30-50 times fold in comparison with the basal activity measured in wild type (WT) plants. When we did the same but transfecting the plants with the DNA construction encoding for AGPPase (regulatory subunit) we observed an increase of activity in respect to the WT 1,5-2 fold. We may conclude, therefore, that new enzyme is an alternative solution, with increased activity expression in transgenic plants, to the AGPPase previously disclosed in the prior art.

We respectfully request to have an additional opportunity to submit further amendments whether the examiner, in view of present answer, he will still maintain any objection or he might raise a new one, about the patentability requirements of the IA.

We also invite to the examiner to have as many informal communication with us as he may consider appropriate to further discuss any outstanding problem or objection the present set of amended claims may have.

Yours faithfully,

ELZABURU

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Manuel Illescas

Enclosures:
- Amended set of claims 1-33

CLAIMS

1.- Enzyme product of plant origin designated NPPase, characterized in that its sequence contains at least one of the polypeptide fragments represented by SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12; SEQ ID NO: 13; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16 and SEQ ID NO: 17 and shows phosphodiesterase activity.

2.- Enzyme product designated NPPase, according to claim 1 characterised by having a amino acid sequence deduced from a cDNA selected among SEQ ID NO: 20 or SEQ ID NO: 22.

3.- Enzyme product, according to claim 2 characterized in that it contains a sequence represented by SEQ ID NO: 21.

4.- Enzyme product, according to claim 2 characterized in that it contains a sequence represented by SEQ ID NO: 23.

5.- Enzyme product according to any of the claims 1 to 4, characterized in that catalyses the hydrolysis of nucleotide sugars in equimolar conditions to sugar-phosphate and the corresponding nucleoside monophosphate, does not hydrolyse molecules with phosphomonoester bonds, is able to hydrolyse bis-PNPP, is inhibited by molybdate, arsenate and phosphorylated molecules, its activity is not affected by reducing and chelating agents that are inhibitors of phosphodiesterases, it is sensitive to slightly basic pH and is very stable at pH between 4 and 7.5, can be glycosylated, which makes it resistant to ionic detergents of the SDS type and to the action of proteases, and recognizes, in addition to nucleotide sugars, other small molecules that possess phosphodiester and phosphosulphate bonds.

6.- Enzyme product as claimed in any of the claims 1 to 5 characterized in that it does not hydrolyse, among others, G1P, G6P, AMP, 3-phosphoglycerate, AMPc, nor nucleic acids.

ART 34 AMDT

7.- Enzyme product as claimed in either one of the claims 1 and 6, characterized in that it does not require as effectors, among other divalent cations, magnesium, manganese or cobalt.

8.- Enzyme product as claimed in any one of the claims 1 to 7, characterized in that it is inhibited by orthophosphate, inorganic pyrophosphate, and phosphate esters such as, among others, AMP, ADP, ATP, or 3-phosphoglycerate.

9.- Enzyme product as claimed in any one of the claims 1 to 8, characterized in that its activity is not affected by, among others, β-mercaptoethanol, EDTA, reduced cysteine or ascorbate.

10.- Enzyme product as claimed in any one of the claims 1 to 9, characterized in that it is resistant to, among others, Proteinase K or Pronase.

11.- Enzyme product as claimed in any one of the claims 1 to 10, characterized in that it recognizes as substrates, among others, ADPG, UDPG, GDP-glucose, ADP-mannose, APS, PAPS or bis-PNPP, the preferred substrate being ADPG.

12.- Enzyme product as claimed in any one of the claims 1 to 11, characterized in that it is resistant to a temperature of 65°C for 30 minutes, and in that it has an apparent molecular weight determined by gel filtration around 70 and 270 kDa for the monomeric and homopolymeric isoform respectively, as well as displaying a K_{eq}' of the reaction of 110, its □G' being -2.9 kcal/mol, and its K_m for ADPG being 0.5 mMolar.

13.- Enzyme product as claimed in any one of the claims 1 to 12, characterized in that it was isolated from any plant species.

14.- Method of obtaining an enzyme product of plant origin with nucleotide sugar pyrophosphatase/phospho-diesterase activity (NPPase) in its soluble isoform, having an amino acid sequence that contains at least one of the polypeptide fragments represented by SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 10; SEQ ID NO: 11; SEQ ID NO: 12; SEQ

ID NO: 13; SEQ ID NO: 14; SEQ ID NO: 15; SEQ ID NO: 16 and SEQ ID NO: 17, characterized in that the material of plant origin is submitted to extraction of the protein fraction by a buffer, filtration of the extract, followed by a method of purification by successive centrifugations and precipitations, with adjustments both of the pH and of the ionic strength of the medium, preferably including heating of the protein above 60°C and cooling in ice, and purification by gel filtration, isoelectric focusing, denaturing-gel electrophoresis, or other equivalent means of purification of proteins extracted from plant tissues.

15.- Method as claimed in claim 14 comprising the following steps: (1) homogenization of the plant tissue with an extraction buffer, type Mes 50 mM pH 6, EDTA 1 mM, DTT 2 mM, (2) filtration, (3) ultracentrifugation at 100 000 *g*, (4) precipitation of the proteins of the supernatant in ammonium sulphate, (5) resuspension of the precipitate in buffer of pH 4.2, (6) heating for at least 15 minutes at a temperature between 60 and 65°C, followed by cooling in ice, (7) centrifugation at 30 000 *g*, (8) concentration of the protein of the supernatant by precipitation in ammonium sulphate and resuspension at pH 6, and (9) purification by gel-filtration chromatography, isoelectric focusing and denaturing-gel electrophoresis.

16.- Primers represented by SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 24.

17.- Use of primers represented by SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 24 together with an mRNA from leaves of rice or barley for obtaining, by RT-PCR, a cDNA which, after being used as a probe on cDNA libraries of leaves of rice and barley, permits the isolation of cDNA's whose sequences are represented by SEQ ID NO: 20 and SEQ ID NO: 22, respectively.

18.- cDNA represented by SEQ ID NO: 20 that codes for an enzyme product with NPPase activity.

19.- Use of primers represented by SEQ ID NO: 18, SEQ ID NO: 19 or SEQ ID NO: 24 together with an mRNA from barley leaves for obtaining, by RT-PCR, a cDNA which, after being used as a probe on cDNA libraries of barley

leaves, permits the isolation of cDNA whose sequence is represented by SEQ ID NO: 22.

20.- cDNA represented by SEQ ID NO: 22 that codes for an enzyme product with NPPase activity.

21.- Use of the enzyme product of claims 1 to 13 in the preparation of assay devices and/or compositions for application in the determination of nucleoside diphosphate sugars.

22.- An assay device for the determination of nucleoside diphosphate sugars, characterized in that it includes the enzyme product of claims 3 to 13 and 19 or 20 in such a way that the determination is based on the sugar-1-phosphate released during the reaction catalysed by NPPase.

23.- The assay device as claimed in claim 22, characterized in that the determination is based on the glucose-1-phosphate released, which is submitted to the enzyme phosphoglucomutase to produce glucose-6-phosphate, which in its turn is submitted to a coupled reaction with NAD^+ and $NADP^+$, by the action of the enzyme glucose-6-phosphate dehydrogenase, obtaining 6-phosphogluconate and NADH or NADPH, either of which can be determined by conventional spectrophotometric methods or methods of some other kind.

24.- An assay device for the determination of nucleoside diphosphate sugars, characterized in that it includes the enzyme product of claims 1 to 13, in such a way that the determination is based on the nucleoside monophosphate produced during the reaction catalysed by NPPase.

25.- The assay device as claimed in claim 24, characterized in that the determination is based on the nucleoside monophosphate, which is able to release orthophosphate, in addition to the corresponding base, by the action of an enzyme such as 5'-nucleotidase, the orthophosphate being easily determined by conventional methods, for example colorimetric methods.

26.- The assay device as claimed in any of the claims 22 to 25, characterized in that the determination is based on the fact that the sugar-1-phosphate and the nucleoside-monophosphate are able to release orthophosphate

by the action of an enzyme such as alkaline phosphatase or 5'-nucleotidase, the orthophosphate being easily determined by conventional methods, for example colorimetric methods.

27.- Use of the enzyme product of claims 1 to 13 in the preparation of assay devices and/or compositions for application in the determination of the presence of 3'-phospho-adenosine 5'-phosphosulphate (PAPS) and adenosine 5'phosphosulphate (APS).

28.- Use of the primers of claims 16 and the cDNA's of claims 18 or 20 in the production of transgenic plants that express or overexpress the cDNA that codes for NPPase.

29.- Method of production of transgenic plants that express or overexpress the gene that codes for NPPase, characterized in that a transformation vector is used that contains a plasmid that includes the cDNA represented by SEQ ID NO: 20 of the gene of the said NPPase.

30.- Method of production of transgenic plants that express or overexpress the gene that codes for NPPase, characterized in that it uses a transformation vector that contains a plasmid that includes the cDNA represented by SEQ ID NO: 22 of the gene of the said NPPase.

31.- A method of production of transgenic plants as claimed in claim 29 or 30, characterized in that the transformation vector is *Agrobacterium tumefaciens* CECT 5799.

32.- Transgenic plants obtainable by the method as claimed in claims 29 to 31, characterized in that they express or overexpress the enzyme product of claims 1 to 13 and have a reduced content of starch and/or of cell-wall polysaccharides and are resistant to high temperatures and to high salinity.

33.- An assay device for the determination of sulphonucleotides, characterized in that it includes the enzyme product of claims 1 to 13 in such a way that the determination is based on the sulphate that is released.

ART 34 AMDT